

A Mammalian Mediator Subunit that Shares Properties with *Saccharomyces cerevisiae* Mediator Subunit Cse2*[§]

Received for publication, November 16, 2003
Published, JBC Papers in Press, November 24, 2003, DOI 10.1074/jbc.M312523200

Chieri Tomomori-Sato^{‡§}, Shigeo Sato^{‡§}, Tari J. Parmely[‡], Charles A. S. Banks[‡], Irina Sorokina[‡], Laurence Florens[‡], Boris Zybailov[‡], Michael P. Washburn[‡], Christopher S. Brower[¶], Ronald C. Conaway^{‡||}, and Joan Weliky Conaway^{‡||***‡}

From the [‡]Stowers Institute for Medical Research, Kansas City, Missouri 64110, the [¶]Division of Biology, California Institute of Technology, Pasadena, California 91125, the ^{||}Department of Biochemistry and Molecular Biology, Kansas University Medical Center, Kansas City, Kansas 66160, and the ^{**}Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

The multiprotein Mediator complex is a coactivator required for activation of RNA polymerase II transcription by DNA bound transcription factors. We previously identified and partially purified a mammalian Mediator complex from rat liver nuclei (Brower, C.S., Sato, S., Tomomori-Sato, C., Kamura, T., Pause, A., Stearman, R., Klausner, R.D., Malik, S., Lane, W.S., Sorokina, I., Roeder, R.G., Conaway, J.W., and Conaway, R.C. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 10353–10358). Analysis by tandem mass spectrometry of proteins present in the most highly purified rat Mediator fractions led to the identification of a collection of new mammalian Mediator subunits, as well as several potential Mediator subunits including a previously uncharacterized protein encoded by the FLJ10193 open reading frame. In this study, we present direct biochemical evidence that the FLJ10193 protein, which we designate Med25, is a *bona fide* subunit of the mammalian Mediator complex. In addition, we present evidence that Med25 shares structural and functional properties with *Saccharomyces cerevisiae* Mediator subunit Cse2 and may be a mammalian Cse2 ortholog. Taken together, our findings identify a novel mammalian Mediator subunit and shed new light on the architecture of the mammalian Mediator complex.

A mammalian Mediator complex similar to yeast Mediator was subsequently identified in several laboratories and designated mouse Mediator (2), TRAP (thyroid hormone receptor-associated proteins)/SMCC (Srb-Med-containing cofactor) (3), ARC (activator-recruited cofactor) (4), DRIP (vitamin D receptor-interacting proteins) (5), CRSP (cofactor required for Sp1 activation) (6), or rat Mediator (7, 8). Biochemical characterization of the mammalian Mediator complex has revealed that it is composed of apparent homologs of many of the *S. cerevisiae* Mediator subunits including TATA-binding protein related factor-proximal protein (TRFP) (Srb2), TRAP80 (Srb4), p28b (Srb5), Surf5 (Srb6), Srb7, TRAP240 (Srb8), TRAP230 (Srb9), Cdk8 (Srb10), Cyclin C (Srb11), TRAP36 (Med4), Med6, Med7, Med8, HSPC296 (Med11), LCMR1 (Rox3), Nut2, Sur2 (Gal11), TRAP95 (Sin4), and Rgr1 (reviewed in Ref. 9).

In a previous study (7), we partially purified a mammalian Mediator complex from rat liver nuclei. Analysis of proteins present in our most highly purified rat Mediator fractions by tandem mass spectrometry led to the identification of a large fraction of the known mammalian Mediator subunits, as well as a collection of potentially new Mediator subunits. In this report, we identify one such protein encoded by the FLJ10193 open reading frame as a new subunit of the mammalian Mediator complex.

EXPERIMENTAL PROCEDURES

Materials—Anti-FLAG (M2) monoclonal antibodies, anti-Myc (C-3956) rabbit polyclonal antibodies, anti-FLAG (M2) agarose, and FLAG peptide were purchased from Sigma. Anti-Myc (9E10) monoclonal antibodies were obtained from Roche Applied Science. Anti-TRAP220 (C-19), anti-Cdk8 (D-9), and anti-Med6 (E-20) antibodies were obtained from Santa Cruz Biotechnology. Anti-Med8 rabbit polyclonal antibodies were raised against a peptide corresponding to Med8 residues 247–268 (Cocalico Biologicals, Inc.). Anti-TRAP80, anti-LCMR1, anti-Intersex, anti-TRAP36, anti-p28b, anti-Surf5, anti-HSPC296, and anti-Med25 rabbit polyclonal antibodies were raised against recombinant proteins expressed in insect cells or *Escherichia coli* (Cocalico Biologicals, Inc.). Light chain-specific anti-mouse antibodies were purchased from Bethyl Laboratories and labeled with Alexa Fluor 680 (Molecular Probes) according to the manufacturer's instructions. SuperSignal West Dura extended duration substrate and SuperSignal West Femto Maximum Sensitivity Substrate were obtained from Pierce. Glutathione-Sepharose 4 Fast Flow and Glutathione-Sepharose 4B beads were from Amersham Biosciences.

Anti-FLAG Agarose Chromatography—Anti-FLAG agarose immunofluorescence chromatography was carried out essentially as described for purification of the TRAP-SMCC Mediator complex (10). HeLa cell nuclear extracts were prepared according to the method of Dignam *et al.* (11). Undialyzed nuclear extracts were incubated with anti-FLAG (M2) agarose beads in 10 mM Hepes-NaOH (pH 7.9), 1.5 mM MgCl₂, 0.3 M KCl, and 0.2% Triton X-100 for at least 4 h at 4 °C. The beads were washed 5 times with a 50-fold excess of the same buffer and once with

The Mediator (Med)¹ is a multiprotein coactivator that promotes RNA polymerase II transcriptional activation through direct interactions with transcription factors bound at enhancers and upstream promoter elements and with polymerase and the general initiation factors at the core promoter. The Mediator was first identified in *Saccharomyces cerevisiae* and found to be comprised of more than twenty proteins including Srb2, Srb4, Srb5, Srb6, Srb7, Srb8, Srb9, Srb10, Srb11, Med1, Med2, Med4, Med6, Med7, Med8, Med11, Pgd1, Rox3, Cse2, Nut1, Nut2, Gal11, Sin4, and Rgr1 (1).

* This work was supported by National Institutes of Health Grant R37 GM41628. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental tables.

[¶] These authors contributed equally to this work.

^{‡‡} To whom correspondence should be addressed. Tel.: 816-926-4091; Fax: 816-926-2093; E-mail: jlc@stowers-institute.org.

¹ The abbreviations used are: Med, Mediator; GST, glutathione S-transferase; MS/MS, tandem mass spectrometry; SMCC, Srb-Med-containing cofactor; Srb, suppressor of RNA polymerase B; TRAP, thyroid hormone receptor-associated protein; HPLC, high pressure liquid chromatography; Ni-NTA, nickel-nitrilotriacetic acid.

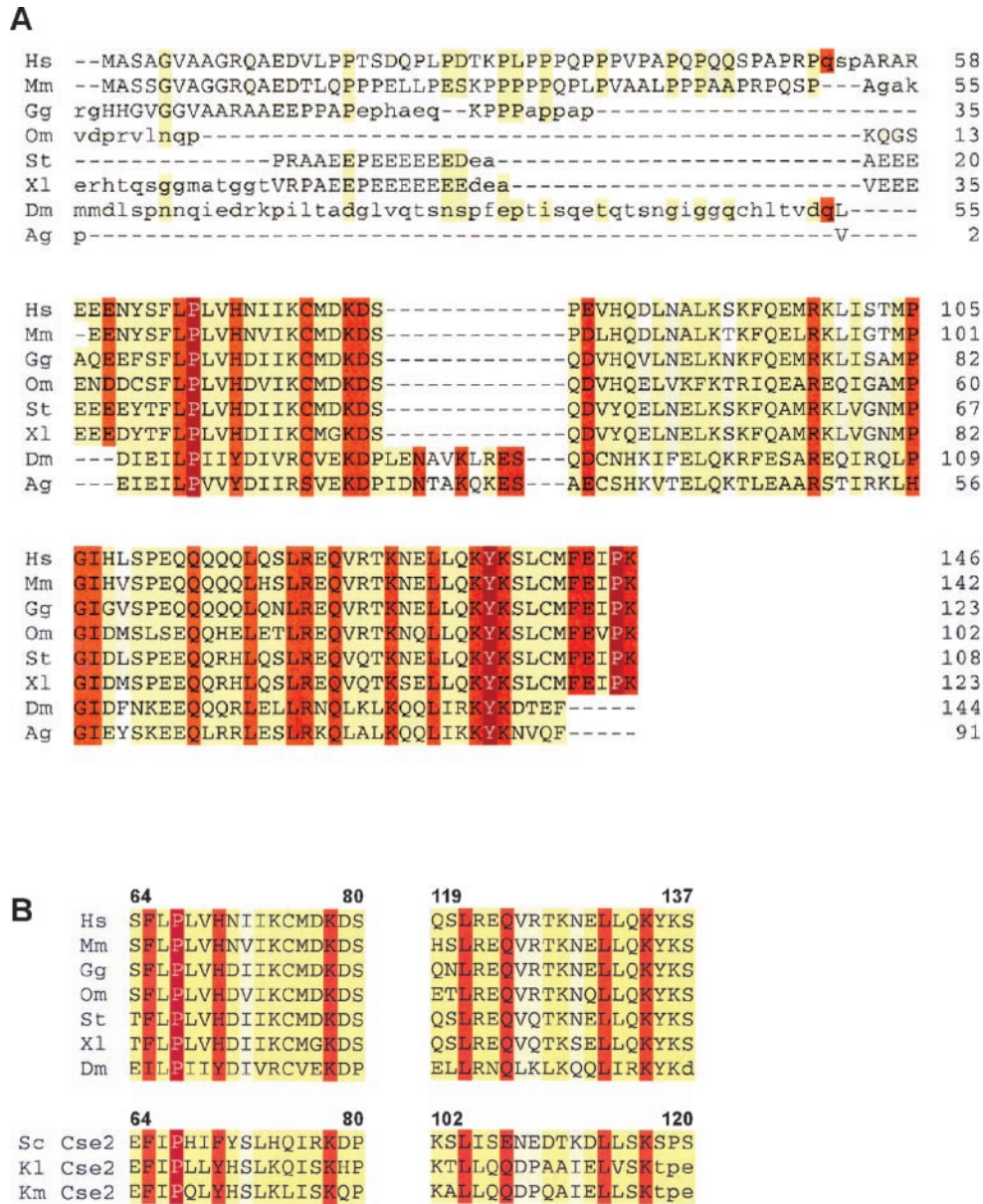


FIG. 1. Sequence of Med25 and similarity to yeast Cse2. Multiple sequence alignments were performed using the MACAW program (18). *Panel A*, multiple sequence alignment of Med25 from higher eukaryotes. Accession numbers are NP_060489, human (*Hs*); NP_619616, mouse (*Mm*); BU228883, *Gallus gallus* (*Gg*); CA346206, *Oncorhynchus mykiss* (*Om*); AL650821, *Silurana tropicalis* (*St*); BF426635, *Xenopus laevis* (*Xl*); CG5134, *Drosophila melanogaster* (*Dm*); AJ285681, *Anopheles gambiae* (*Ag*). *Panel B*, similarity of Med25 to yeast Cse2. Accession numbers for higher eukaryotic Med25 orthologs are given under *panel A*. Accession numbers for yeast Cse2 orthologs are YNR010w, *S. cerevisiae* (*Sc*); AL425926, *Kluyveromyces lactis* (*Kl*); AL424134, *Kluyveromyces marxianus* (*Km*).

a 50-fold excess of 10 mM Hepes-NaOH (pH 7.9), 1.5 mM MgCl₂, 0.1 M NaCl, and 0.05% Triton X-100. Beads were then eluted with 10 mM Hepes-NaOH (pH 7.9), 1.5 mM MgCl₂, 0.1 M NaCl, and 0.05% Triton X-100, and 0.2 mg/ml FLAG peptide. All buffers contained 1:1000-fold dilution of Protease Inhibitor Mixture (Sigma catalog number P8340).

Mass Spectrometry—Identification of proteins was accomplished using a modification of the MudPIT procedure described by Washburn *et al.* (12). Aliquots of anti-FLAG agarose eluates were brought to 100 μl with 1 M Tris-HCl (pH 8.5) and H₂O to a final concentration of 100 mM Tris-HCl. Solid urea was added to 8 M. Proteins were reduced with 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Roche), alkylated with 20 mM iodoacetamide (IAM, Sigma), and digested with endoproteinase Lys-C (Roche) for at least 6 h at 37 °C. Proteins were then diluted 4 times 100 mM Tris-HCl (pH 8.5) and digested overnight at 37 °C with modified Trypsin (Roche) in the presence of 2 mM CaCl₂. Peptide mixtures were pressure-loaded onto a 250 μm inner diameter (i.d.) fused-silica capillary packed first with 4 cm of 5 μm strong cation exchange material (Partisphere SCX, Whatman), followed by 2 cm of 5 μm C18 reverse phase particles (Aqua, Phenomenex). Loaded and washed microcapillaries were connected *via* a 2 μm filtered union

(UpChurch Scientific) to a 100 μm i.d. column, which had been pulled to a 5 μm i.d. tip using a P-2000 CO₂ laser puller (Sutter Instruments), then packed with 10 cm of reverse phase particles and equilibrated in 5% acetonitrile, 0.1% formic acid (Buffer A). This split-column was then installed in-line with a Quaternary Agilent 1100 series HPLC pump. Overflow tubing was used to decrease the flow rate from 0.1 ml/min to about 200–300 nl/min. Fully automated 6 step chromatography runs were carried out. Three different elution buffers were used: Buffer A; 80% acetonitrile, 0.1% formic acid (Buffer B); and 0.5 M ammonium acetate, 5% acetonitrile, 0.1% formic acid (Buffer C). In such sequences of chromatographic events, peptides are sequentially eluted from the SCX resin to the reverse phase resin by increasing salt steps (increase in Buffer C concentration), followed by organic gradients (increase in Buffer B concentration). The last chromatography step consists in a high salt wash with 100% Buffer C followed by the acetonitrile gradient. The application of a 2.5 kV distal voltage electrosprayed the eluting peptides directly into a Deca-XP ion trap mass spectrometer equipped with a nano-LC electrospray ionization source (ThermoFinnigan). Full MS spectra were recorded on the peptides over a 400 to 1,600 *m/z* range, followed by three tandem mass (MS/MS) events sequentially generated

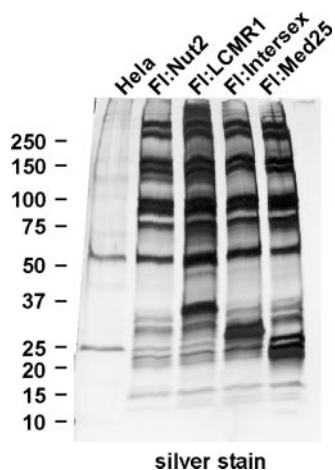


FIG. 2. SDS-PAGE of mammalian Mediator preparations used in this study. Anti-FLAG agarose immunoaffinity chromatography of nuclear extracts prepared from equivalent numbers of HeLa cells and FLAG-Nut2, FLAG-LCMR1, FLAG-Intersex, and FLAG-Med25 expressing HeLa cells was carried out as described under "Experimental Procedures." Aliquots of anti-FLAG agarose eluates were analyzed by SDS-PAGE in a 4–20% gradient gel, and proteins were visualized by silver staining. The positions and relative molecular masses in kDa of protein size standards are indicated at the left of the gel. *HeLa*, anti-FLAG agarose eluate from parental HeLa cells; *Fl:Nut2*, FLAG-Nut2; *Fl:LCMR1*, FLAG-LCMR1; *Fl:Intersex*, FLAG-Intersex; *Fl:Med25*, FLAG-Med25.

in a data-dependent manner on the first, second, and third most intense ions selected from the full MS spectrum (at 35% collision energy). Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (ThermoFinnigan). The software algorithm 2to3 (13) was used to determine charge state and to delete spectra of poor quality. SEQUEST (14) was used to match MS/MS spectra to peptides in a data base containing 82,422 human, mouse, and rat protein sequences downloaded from NCBI on October 23, 2003. The validity of peptide/spectrum matches was assessed using the SEQUEST-defined parameters, cross-correlation score (XCORR) and normalized difference in cross-correlation scores (DeltCn). Spectra/peptide matches were only retained if they had a DeltCn of at least 0.08 and minimum XCORR of 1.8 for +1, 2.5 for +2, and 3.5 for +3 spectra. In addition, the peptides had to be at least 7 amino acids long. DTASelect (15) was used to select and sort peptide/spectrum matches passing this criteria set. Peptide hits from multiple runs were compared using CONTRAST (15).

RESULTS AND DISCUSSION

We previously described partial purification from rat liver nuclear extracts of a multiprotein Mediator complex exhibiting an apparent native molecular mass by gel filtration of more than 1000 kDa (7). Our most highly purified rat Mediator contained many previously characterized mammalian Mediator subunits, as well as a collection of potential Mediator subunits including the p28b, Surf5, LCMR1, HSPC296, and mammalian Intersex proteins, which we subsequently demonstrated are *bona fide* subunits of the mammalian Mediator complex (8, 16). Among the collection of additional proteins present in our most highly purified rat Mediator fractions and identified by mass spectrometry was a 142-amino acid protein encoded by the FLJ10193 open reading frame (Fig. 1A). PSI-BLAST searches of the NCBI protein data base indicates that the FLJ10193 protein has diverged substantially during evolution, with obvious FLJ10193 orthologs in insects, but not in more distantly related species such as *Caenorhabditis elegans* and yeast. The N-terminal ~50 amino acids of FLJ10193 diverge significantly even between human and mouse; in contrast, the C-terminal ~80 amino acids is highly conserved from mammals to insects, suggesting that it may represent the functional core of the protein.

Multiple sequence alignments of the FLJ10193 protein with

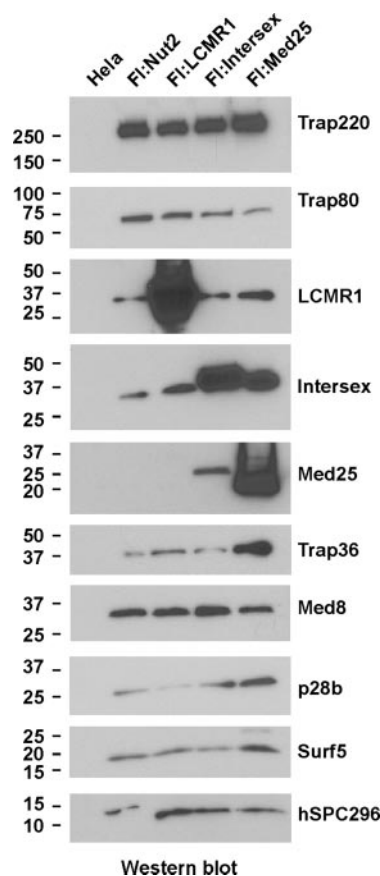


FIG. 3. The Med25 protein is present in the purified TRAP-SMCC Mediator complex. Anti-FLAG agarose immunoaffinity chromatography of nuclear extracts from HeLa cells or FLAG-Nut2, FLAG-LCMR1, FLAG-Intersex, and FLAG-Med25 expressing HeLa cells was carried out as described under "Experimental Procedures." Aliquots of anti-FLAG eluates were fractionated by SDS-PAGE electrophoresis in 4–20% gradient gels, and proteins were visualized by Western blotting with antibodies recognizing the Mediator subunits indicated in the figure. Western blots were developed using horseradish peroxidase-labeled secondary antibodies and Super-Signal West Dura extended duration substrate. The positions and relative molecular masses in kDa of protein size standards are indicated at the left of the blots. *HeLa*, anti-FLAG agarose eluate from parental HeLa cells; *Fl:Nut2*, FLAG-Nut2; *Fl:LCMR1*, FLAG-LCMR1; *Fl:Intersex*, FLAG-Intersex; *Fl:Med25*, FLAG-Med25.

yeast Mediator subunits identified two short regions of FLJ10193 with some similarity to *S. cerevisiae* Mediator subunit Cse2 (Fig. 1B), raising the possibility that FLJ10193 and Cse2 may be related. Notably, like the sequence of the FLJ10193 protein, the sequence of Cse2 has diverged substantially during evolution, with apparent Cse2 orthologs in yeasts closely related to *S. cerevisiae*, but not in more distantly related yeasts such as *Schizosaccharomyces pombe*, *Candida*, and *Neurospora*. Below we present direct biochemical evidence that the FLJ10193 protein, which we designate Med25, is a previously unrecognized subunit of the mammalian Mediator complex with features similar to those of yeast Mediator subunit Cse2.

To begin to address the possibility that the Med25 protein is a component of the mammalian Mediator complex, we sought to identify Med25-associating proteins. To accomplish this, we took advantage of four HeLa cell lines stably expressing either human Mediator subunit Nut2 (10), mouse Mediator subunit LCMR1 (16), human Mediator subunit Intersex (16), or the mouse Med25 protein, all with N-terminal FLAG epitope tags. The FLAG-Nut2 expressing cell line has been used extensively for preparation of the transcriptionally active TRAP/SMCC Mediator complex (10), and we have previously used HeLa cell

lines expressing FLAG-tagged mouse LCMR1 and FLAG-tagged human Intersex as sources for anti-FLAG immunofluorescence purification of the mammalian Mediator complex (16).

Nuclear extracts prepared from equivalent numbers of parental, FLAG-Nut2, FLAG-LCMR1, FLAG-Intersex, and FLAG-Med25 expressing HeLa cells were subjected to anti-FLAG agarose chromatography as described previously for purification of the TRAP-SMCC Mediator complex (10). As shown

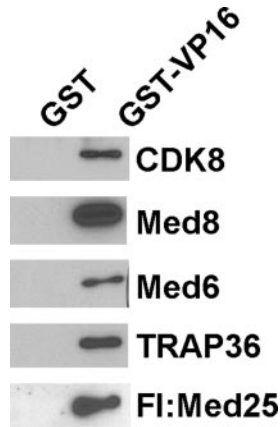


FIG. 4. The Med25 protein associates with the VP16 transcriptional activation domains. GST-VP16 or GST alone was prebound to glutathione-Sepharose 4B beads in 10 mM Hepes-NaOH (pH 7.9), 0.3 M KCl, 1.5 mM MgCl₂, 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, and 0.1% Triton X-100 and incubated for at least 4 h at 4 °C with undialyzed nuclear extracts prepared from FLAG-Med25 expressing HeLa cells according to the method of Dignam *et al.* (11). The glutathione-Sepharose beads were then washed 7 times with a 50-fold excess of the same buffer containing 0.5 M KCl, and bound proteins were eluted from the beads with 100 mM Tris-HCl (pH 7.9), 0.12 M NaCl, 0.1% Triton X-100, and 20 mM glutathione. Aliquots of glutathione-Sepharose eluates were analyzed by 10% or 13% SDS-PAGE, and proteins were visualized by Western blotting with antibodies recognizing the Mediator subunits indicated in the figure. Western blots were developed using horseradish peroxidase-labeled secondary antibodies and Super-Signal West Dura extended duration substrate or SuperSignal West Femto Maximum Sensitivity Substrate.

in the silver-stained SDS-polyacrylamide gel of Fig. 2, anti-FLAG agarose eluate from FLAG-Med25 expressing HeLa cells appeared to include a very similar set of proteins as the TRAP-SMCC Mediator complex purified from FLAG-Nut2, FLAG-LCMR1, or FLAG-hIntersex expressing HeLa cells. In addition, analysis of Med25-associating proteins present in the anti-FLAG agarose eluate from FLAG-Med25 expressing HeLa cells by tandem mass spectrometry (Table I in Supplemental Material) and by Western blotting (Fig. 3) identified nearly all of the mammalian Mediator subunits known to be present in the TRAP-SMCC Mediator complex, arguing that Med25 is a previously unrecognized subunit of the mammalian Mediator complex. The Med25 protein was detected by Western blotting only in anti-FLAG agarose eluates from FLAG-Med25 and FLAG-Intersex expressing HeLa cells. Med25 was, however, detected by tandem mass spectrometry in anti-FLAG-agarose eluates from all four HeLa stable cell lines, but not from parental HeLa cells (Table II in Supplemental Material). These observations suggest that the Med25 protein is present at varying levels in these different Mediator preparations. Finally, and consistent with previous findings indicating that the mammalian Mediator complex binds to and can be purified through interactions with the VP16 transcriptional activation domain (4, 10), we observe that the Med25 protein can be purified together with other Mediator subunits from nuclear extracts of FLAG-Med25 expressing HeLa cells by GST-VP16 chromatography. As shown in the Western blot of Fig. 4, the Med25 protein and representative Mediator subunits Cdk8, Med8, Med6, and TRAP36 are copurified from HeLa cell nuclear extracts using immobilized GST-VP16, but not GST. Thus, the Med25 protein copurifies with the mammalian Mediator complex by several independent methods, arguing that it is a *bona fide* Mediator subunit.

To obtain additional evidence supporting assignment of the Med25 protein as a new subunit of the mammalian Mediator complex, we sought to identify pair-wise binding partners of Med25 among the known mammalian Mediator subunits. To accomplish this, we began by exploiting a convenient screen (8)

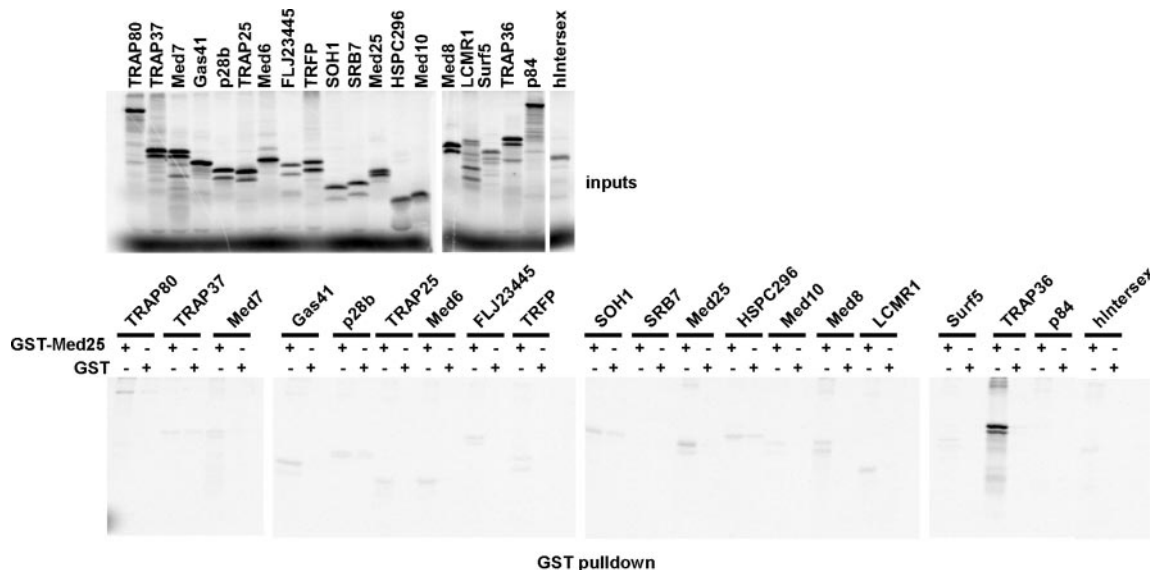


FIG. 5. Screening for Med25 binding partners among the known mammalian mediator subunits. ³⁵S-labeled Mediator subunits were synthesized in the rabbit reticulocyte lysate TNT T7 Quick Coupled Transcription/Translation System (Promega) programmed with pcDNA3.1 expression vectors encoding the mammalian Mediator subunits and other proteins indicated in the figure. Reticulocyte lysates containing the indicated ³⁵S-labeled proteins were mixed with bacterially expressed and purified GST-Med25 or GST alone prebound to glutathione-Sepharose 4 Fast Flow beads in phosphate-buffered saline and incubated for 2 h with rocking at 4 °C. The beads were washed 2 times with 40 mM Tris-HCl (pH 7.5), 0.25 M NaCl, and 0.2% Triton X-100. The beads were then washed once with Tris-buffered saline, and proteins were eluted from the beads with Tris-buffered saline containing 10 mM glutathione. Aliquots of glutathione-Sepharose eluates were fractionated by 12% SDS-PAGE, and proteins were visualized using a Typhoon 8600 PhosphorImager (Amersham Biosciences).

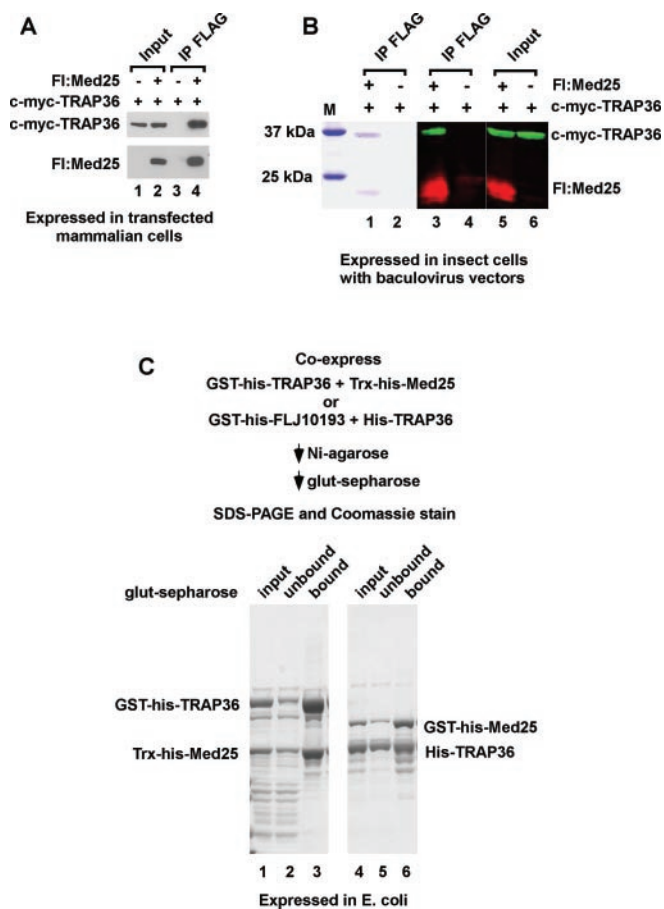


FIG. 6. Interaction of Med25 with the TRAP36/Med4 subunit of the mammalian Mediator complex. *Panel A*, interaction of the Med25 and TRAP36 proteins in transfected human 293T cells. Two 10-cm dishes of 293T cells grown to ~50% confluency were cotransfected with 3 μ g of pcDNA3.1 expression vectors encoding the Med25 protein with an N-terminal FLAG tag and the TRAP36 protein with an N-terminal Myc tag. 24–36 h after transfection, cells were lysed by incubation in 50 mM Hepes-NaOH (pH 7.9), 0.25 M KCl, 0.2% Triton X-100, and 20% (v/v) glycerol. The lysates were subjected to anti-FLAG agarose chromatography as described under “Experimental Procedures,” and proteins present in anti-FLAG agarose eluates were fractionated by SDS-PAGE and visualized by Western blotting with anti-Myc and anti-Med25 antibodies using the Super-Signal West Dura extended duration substrate. *Panel B*, interaction of the Med25 and TRAP36 proteins in Sf21 insect cells. Sf21 cells were cultured at 27 °C in Sf-900 II SFM (Invitrogen) with 10% fetal calf serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Sf21 cells were infected at a multiplicity of infection of 10 with recombinant baculoviruses encoding the Med25 protein with an N-terminal FLAG tag and the TRAP36 protein with an N-terminal Myc tag. Forty eight h after infection, cells were collected and lysed in ice-cold buffer containing 50 mM Hepes-NaOH (pH 7.9), 0.5 M NaCl, 5 mM MgCl₂, 0.2% Triton X-100, 20% (v/v) glycerol, 0.28 μ g/ml leupeptin, 1.4 μ g/ml pepstatin A, 0.17 mg/ml phenylmethylsulfonyl fluoride, and 0.33 mg/ml benzimidazole. Lysates were centrifuged 20,000 \times g for 30 min at 4 °C and proteins were subjected to immunoprecipitation with anti-FLAG agarose, and bound proteins were eluted from anti-FLAG beads with 150 μ g/ml FLAG peptide. Proteins present in anti-FLAG agarose eluates were fractionated by SDS-PAGE and visualized by Coomassie staining (*lanes 1 and 2*) or by Western blotting (*lanes 3–6*). FLAG-Med25 was detected with anti-FLAG (M2) monoclonal antibodies and Alexa Fluor 680-labeled anti-mouse IgG (α light chain-specific) secondary antibodies (*red*); Myc-TRAP36 was detected with rabbit anti-Myc antibodies and IR DyeTM 800-labeled goat anti-rabbit IgG secondary antibodies (*green*). Fluorescently labeled secondary antibodies were detected using a Li-Cor Odyssey infrared imaging system. *Panel C*, interaction of the Med25 and TRAP36 proteins in *E. coli*. Either GST-his-TRAP36 and thioredoxin (Trx)-his-Med25 fusion proteins or GST-his-Med25 and his-TRAP36 fusion proteins were coexpressed in *E. coli* BL21(DE3) cells. Cell lysates were fractionated by Ni²⁺ chromatography using Ni-NTA agarose (Qiagen) equilibrated in buffer containing 50 mM Tris-HCl (pH 7.9), 0.3 M NaCl, 10% (v/v) glycerol, 0.1% Triton X-100, protease inhibitor mixture

to assess the ability of Med25 to interact with known mammalian Mediator subunits prepared by *in vitro* translation in rabbit reticulocyte lysates. pcDNA3.1 expression vectors encoding the known mammalian Mediator subunits and additional proteins indicated in Fig. 5 were used to program rabbit reticulocyte lysates for translation of ³⁵S-labeled Mediator proteins. Binding of ³⁵S-labeled Mediator proteins to a GST-Med25 fusion protein was assayed in GST pull-down experiments using glutathione-agarose beads, and bound proteins were visualized by autoradiography. The results of this screen revealed significant and reproducible binding of Med25 to Mediator subunit TRAP36 (Fig. 5), the mammalian ortholog of yeast Mediator subunit Med4. Notably, in a previous study, *S. cerevisiae* Mediator subunit Cse2 was found to interact directly only with yeast Mediator subunit Med4 (17), suggesting that Med25 and Cse2 may be functionally related.

To confirm and extend this finding, we sought to reconstitute the Med25-TRAP36 interaction detected in *in vitro* translation experiments in transiently transfected human 293T cells and with recombinant proteins expressed in Sf21 insect cells and *E. coli*. As shown in Fig. 6A, the TRAP36 protein could be coimmunoprecipitated with the Med25 protein from lysates of 293T cells cotransfected with pcDNA3.1 expression vectors encoding TRAP36 and Med25. In addition, direct pairwise binding of Med25 to TRAP36 was detected following their coexpression in Sf21 cells (Fig. 6B) and in *E. coli* (Fig. 6C), arguing that Med25 binds directly to TRAP36 and may be recruited to the Mediator complex through this interaction.

In summary, in this report we identify the previously uncharacterized FLJ10193 protein as a new mammalian Mediator subunit designated Med25. Although lower eukaryotic orthologs of the Med25 protein have not yet been unequivocally identified, our findings have brought to light structural and functional similarities between Med25 and yeast Mediator subunit Cse2. First, Med25 exhibits limited sequence similarity with Cse2. Second, like Cse2, the Med25 protein forms a heterodimer with Mediator subunit Med4 (or TRAP36 in mammals) and thus may be recruited to the “middle module” of the mammalian Mediator complex through its interaction with Med4. Whether Med25 fulfills a similar role as Cse2 in Mediator, however, remains to be determined.

Acknowledgments—We thank W. S. Lane for assistance with mass spectrometry. We also thank R. G. Roeder and S. Malik for the HeLa cell line stably expressing FLAG-Nut2.

REFERENCES

- Myers, L. C., and Kornberg, R. D. (2000) *Annu. Rev. Biochem.* **69**, 729–749
- Jiang, Y., Veschambre, P., Erdjument-Bromage, H., Tempst, P., Conaway, J. W., Conaway, R. C., and Kornberg, R. D. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 8538–8543
- Gu, W., Malik, S., Ito, M., Yuan, C. X., Fondell, J. D., Zhang, X., Martinez, E., Qin, J., and Roeder, R. G. (1999) *Mol. Cell* **3**, 97–108
- Naar, A. M., Beaurang, P. A., Zhou, S., Abraham, S., Solomon, W., and Tjian, R. (1999) *Nature* **398**, 828–832
- Rachez, C., Lemon, B. D., Suldan, Z., Bromleigh, V., Gamble, M., Naar, A. M., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1999) *Nature* **398**, 824–827
- Ryu, S., Zhou, S., Ladurner, A. G., and Tjian, R. (1999) *Nature* **397**, 446450

(Sigma), and 20 mM imidazole. Proteins bound to the Ni-NTA agarose were eluted in the same buffer containing 0.3 M imidazole. Ni-NTA eluates were then further fractionated by glutathione-Sepharose chromatography using glutathione-Sepharose 4B pre-equilibrated in Ni-NTA elution buffer lacking imidazole. Following binding of proteins in Ni-NTA agarose eluates to glutathione-Sepharose, the column was washed with Ni-NTA elution buffer lacking imidazole, and proteins were eluted with the same buffer containing 20 mM glutathione and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride. Proteins present in aliquots of glutathione-Sepharose eluates were analyzed by 10% SDS-PAGE and visualized by Coomassie staining. *IP*, immunoprecipitation; *FL:Med25*, FLAG-Med25; *his*, His₆ tag; *Trx*, thioredoxin; *glut*, glutathione.

7. Brower, C. S., Sato, S., Tomomori-Sato, C., Kamura, T., Pause, A., Stearman, R., Klausner, R. D., Malik, S., Lane, W. S., Sorokina, I., Roeder, R. G., Conaway, J. W., and Conaway, R. C. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 10353–10358
8. Sato, S., Tomomori-Sato, C., Banks, C. A., Sorokina, I., Parmely, T. J., Kong, S. E., Jin, J., Cai, Y., Lane, W. S., Brower, C. S., Conaway, J. W., and Conaway, R. C. (2003) *J. Biol. Chem.* **278**, 15123–15127
9. Boube, M., Joulia, L., Cribbs, D. L., and Bourbon, H.-M. (2002) *Cell* **110**, 143–151
10. Malik, S., Gu, W., Wu, W., Qin, J., and Roeder, R. G. (2000) *Mol. Cell* **5**, 753–760
11. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489
12. Washburn, M. P., Wolters, D., and Yates, J. R., III (2001) *Nat. Biotechnol.* **19**, 242–247
13. Sadygov, R. G., Eng, J., Durr, E., Saraf, A., McDonald, H., MacCoss, M. J., and Yates, J. R., III (2002) *J. Proteome Res.* **1**, 211–215
14. Eng, J. K., McCormick, A. L., and Yates, J. R., III (1994) *J. Am. Soc. Mass. Spec.* **5**, 976–989
15. Tabb, D. L., McDonald, W. H., and Yates, J. R., III (2002) *J. Proteome Res.* **1**, 21–26
16. Sato, S., Tomomori-Sato, C., Banks, C. A., Parmely, T. J., Sorokina, I., Brower, C. S., Conaway, R. C., and Conaway, J. W. (2003) *J. Biol. Chem.* **278**, 49671–49674
17. Kang, J. S., Kim, S. H., Hwang, M. S., Han, S. J., Lee, Y. C., and Kim, Y. J. (2001) *J. Biol. Chem.* **276**, 42003–42010
18. Schuler, G. D., Altschul, S. F., and Lipman, D. J. (1991) *Proteins Struct. Funct. Genet.* **9**, 180–190